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Dose-Response Relationships for Experimental Heterochrony in a Colonial Hydroid

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Abstract. Hydractiniid hydroids display a range of morphological variation. At one end of the spectrum, the colonies grow in a sheet-like configuration with their polyps close together and short stolons. At the other extreme, the colonies have a runner-like form in which the polyps are farther apart and connected by long stolons. These patterns exemplify the heterochronic variation found in many colonial animals and correspond to changes in the timing of the production of polyps and stolon tips relative to rates of stolon growth and colony maturation. Experimental studies of clonal replicates of a *Podocoryne carnea* colony demonstrated a dose-response relationship between these heterochronic traits and within-colony gastrovascular flow to peripheral stolons. A dose-response relationship was found whether flow was perturbed by manipulating the amount of food consumed by the colonies or by treating the colonies with 2, 4-dinitrophenol, an uncoupler of oxidative phosphorylation. In colonies in which flow was highly perturbed by either treatment, a similar rate of flow produced a similar morphological response. These data support the hypothesis of a causal relationship between flow rate and heterochronic variation. Nevertheless, flow was diminished by two clearly different mechanisms. Feeding manipulation altered flow relative to the size of the stolon by altering stolon thickness, without affecting the absolute quantity of flow. Uncoupling with dinitrophenol diminished the absolute quantity of flow, but did not affect the size of the stolon. A plausible assumption is that feeding manipulation affects the resistance of the stolon tissue to flow, or the fluid absorption of this tissue, or both; whereas uncoupling affects the amount of energy available to drive the flow. At the level of cellular metabolism, on the other hand, feeding manipulation and uncoupling again have similar effects, triggering metabolic activation (*e.g.*, an increase in oxygen uptake and a shift in the mitochondrial redox state in the direction of oxidation). In the context of theories suggesting the metabolic control of development, a direct effect of feeding and uncoupling on colony development thus cannot be ruled out. Further, there may be an interaction between flow rate and metabolism, since gastrovascular flow distributes food throughout the colony, and since such substrate affects metabolic state. Both within-colony flow rate and metabolism may affect heterochronic variation in these hydroids, and methods appropriate to distinguish these two effects are discussed.

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Introduction

Evolutionary changes in the timing of development underlie much of animal diversity (*e.g.*, Gould, 1977; Alberch *et al.*, 1979; Bonner, 1982; McKinney, 1988; Wake *et al.*, 1991; Hall, 1992). Historically, studies of heterochrony have focused on describing patterns of comparative embryology and morphology (Gould, 1977), and to a large degree this descriptive and correlational tradition still persists (see Raff and Wray, 1989). Thus, relatively little is known about the actual mechanisms that govern heterochronic variation. Are morphological and life-history heterochronies direct consequences of genetic and molecular heterochronies? Do physiological, developmental, and metabolic factors mediate heterochronic variation? Can unrelated genetic changes produce similar heterochronies by affecting the same epigenetic process? Investigations of such questions are crucial if studies of heterochrony are to progress beyond largely descriptive studies of morphology (Raff and Wray, 1989).

To address these sorts of questions, whole-organism experimental manipulations have become increasingly

common in studies of evolutionary morphology (*cf.*, Ketterson and Nolan, 1992; Sinervo *et al.*, 1992; Sinervo and Basalo, 1996), and this is particularly true in studies of the evolution of development (*e.g.*, Alberch and Gale, 1985; Stebbins and Basile, 1986; Meyer, 1987; Müller, 1991; DeSalle and Carew, 1992; Blackstone and Buss, 1992, 1993; Dudgeon and Buss, 1996). The central motivation for these latter studies is articulated by Stebbins and Basile (1986) in their definition of phyletic phenocopies: "We propose this term for changes in form or physiological response that mimic the normal form or reaction of a related phenotype, particularly one belonging to a different taxon. Using them, investigators have obtained clues to the developmental basis of evolutionary change, and occasionally to the nature and action of the genes involved." Experimental aspects of this approach can be particularly effective in the context of studies of heterochrony (*e.g.*, Meyer, 1987). Nevertheless, to definitively investigate the basis for heterochrony, such experiments should include not just gene dosage studies (*e.g.*, DeSalle and Carew, 1992), but also manipulations of physiological and developmental mechanisms against a uniform genetic background. Further, once an experimental basis for heterochrony has been demonstrated, the causal basis of such a relationship should be further elucidated by establishing the underlying biological gradient or dose-response curve (*e.g.*, Hill, 1965; Weed, 1988).

As model systems for experimental studies of the evolution of development, clonal organisms (*e.g.*, many fungi, herbaceous plants, and colonial invertebrates) are particularly appropriate. In such organisms, the growth and development of the colony are inseparable, and there is a broad chronological window in which manipulations of development are possible; moreover, genetically identical clonal replicates can be used in these experiments (Buss and Blackstone, 1991). In many clonal groups, the morphology can be idealized as comprising feeding and reproductive entities, here termed polyps, which are interconnected by vascular stolons. Runner-like forms (*cf.*, "guerrilla" of Harper, 1985) show widely spaced polyps and long stolonal connections, while sheet-like forms (*cf.*, "phalanx" of Harper, 1985) show closely packed polyps with short stolonal connections. These different morphological patterns correspond to changes in the timing of the production of polyps and stolon tips relative to the rates of stolon growth and colony maturation; high rates of production yield sheets, whereas low rates yield runners. Further, these distinctive morphologies correlate with a variety of life-history traits; runner-like forms tend to grow quickly, reproduce early, and disperse widely as compared to sheet-like forms (*e.g.*, Jackson, 1979; Harper, 1985). In the terminology of heterochrony, runner-like forms often exhibit progenesis (*i.e.*, precocious sexual reproduction) and paedomorphic (*i.e.*, "child-shaped") adult morphologies.

Hydractiniid hydroids illustrate these general patterns: species of *Hydractinia* are typically sheet-like, while species of *Podocoryne* are typically runner-like (Blackstone and Buss, 1991; Blackstone, 1996). The morphological aspects of this variation derive from the higher rates of polyp and stolon tip formation, relative to rates of stolon growth and colony maturation, in *Hydractinia* compared to *Podocoryne*. This difference is particularly pronounced at the time of the formation of the stolonal mat in *Hydractinia*. In addition, the relative rates of polyp and stolon production show an inverse correlation with rates of gastrovascular fluid flow to peripheral stolon tips. Compared to colonies of *Podocoryne carnea*, mature colonies of *Hydractinia symbiolongicarpus* exhibit a low rate of flow to peripheral stolons, particularly subsequent to the formation of the stolonal mat (Blackstone and Buss, 1992; Blackstone, 1996).

Experimental studies of heterochrony in these hydroids have demonstrated that the between-species pattern can be mimicked by experimental manipulation of colonies of a single species (Blackstone and Buss, 1992, 1993). Putatively, these hydroids incur substantial energetic costs in circulating the gastrovascular fluid throughout the colony. Application of 2,4-dinitrophenol to colonies of *Podocoryne carnea* results in a condition of "loose-coupling" of oxidative phosphorylation, a decrease in the energy available for generating gastrovascular flow, and a consequent diminishing of the rate of flow to peripheral stolons. Correlated with this diminished flow are changes that parallel patterns of heterochrony; the rate of production of polyps and stolon tips increases relative to the rates of stolon growth and colony maturation, and peramorphic ("shapes beyond") forms result. Alternatively, gastrovascular flow can be diminished by increasing the number of times a colony is fed (*e.g.*, from 3 to 6 times per week), possibly because a higher rate of feeding increases either the viscosity of the gastrovascular fluid or the resistance to flow of the stolonal tissues, or both. Feeding manipulation, like treatment with uncouplers, results in changes that parallel patterns of heterochrony; again, the rate of production of polyps and stolon tips increases relative to rates of stolon growth and colony maturation (see Braverman, 1974). Although increased feeding produces a surfeit of nutrients and seems in many ways the opposite of the energy-poor state produced by uncoupling, its effect on colony physiology (*i.e.*, flow rate) is similar. In combination, the between-species data (Blackstone and Buss, 1992; Blackstone, 1996) and the experimental manipulations (Blackstone and Buss, 1992, 1993) suggest that flow rate is a principal mechanism underlying heterochronic alterations of these hydroid colonies.

Nevertheless, these interpretations are complicated by the similarities potentially induced by feeding manipulation and uncoupling treatments at the level of cellular metabolism (*e.g.*, Chance *et al.*, 1963; Heytler, 1981; Chance, 1991). Briefly, feeding triggers metabolic activation, which has features (*e.g.*, increased oxygen consumption, shift of the mitochondrial redox state in the direction of oxidation) that can be mimicked by uncoupling. In this latter case, oxidation of substrate is "uncoupled" from energy conversion, so metabolic activation does not lead to increased ATP formation. Treatments involving feeding manipulation and uncoupling may thus share some characteristics at the level of mitochondrial metabolism, but diverge at the level of ATP production. Further, the features of metabolic activation in experiments with uncouplers are often sensitive to the nature of the particular uncoupler used, its concentration, and other aspects of the experimental protocols.

Given these considerations, further investigations into the relationship between gastrovascular flow rate and heterochronic variation in these hydroids are needed. Here, two series of experiments are reported. The first series focused on causal criteria: if a dose-response relationship exists between flow rate and heterochronic variation, a causal link between these variables gains considerable support (*e.g.*, Hill, 1965; Weed, 1988). Hence, two dose-response experiments were carried out with clonal replicates of the same *P. carnea* colony. The first experiment examined dose-response in feeding experiments. Although the actual experimental treatment is the number of feedings per week, gastrovascular flow rate (the hypothetical physiological dose) is considered the predictor variable; morphological development relative to the timing of the sexual (medusoid) phase of the life cycle is considered the response variable. The second experiment examined dose-response in uncoupling experiments. Again, gastrovascular flow rate (the hypothetical physiological dose) is considered the predictor variable, and morphological development relative to the timing of the sexual (medusoid) phase of the life cycle is considered the outcome variable. Both experiments support the hypothesis of a dose-response relationship between gastrovascular flow and heterochronic variation in these hydroids.

The second series of experiments directly investigated metabolic characteristics of colonies subjected to feeding manipulation and uncoupling; oxygen uptake and mitochondrial redox state were measured in clonal replicates of the same *P. carnea* colony. The results suggest some parallels between these treatments at the level of cellular metabolism, although the extent to which such features can directly affect colony development remains unexplored. However, these results complicate the interpretation of the dose-response relationships, and these complications, and the methods appropriate for their resolution, are discussed. At this time, it seems possible that both colony physiology (*i.e.*, flow rate) and metabolism may mediate the genetic aspects of heterochrony in these hydroids.

Materials and Methods

Study species

Podocoryne carnea exhibits traits typical of runner-like colonial animals (Blackstone, 1996). Colony development begins with the metamorphosis of the planula larva into a primary polyp. Runner-like stolons extend from the primary polyp. Stolons encase fluid-filled canals that are continuous with the gastrovascular cavity of the polyp. In cross section, stolons consist of a fluid-filled lumen encased by endoderm, ectoderm, and a rigid periderm. Gastrovascular fluid circulates in the lumen of the stolons and carries food and possibly other metabolites from the feeding polyp to other parts of the colony; contractions of the muscular polyp propel the gastrovascular fluid (Schierwater *et al.*, 1992). As the lumen fills and empties in response to contractions of the polyps, the endodermal and ectodermal tissue layers of the stolon expand and contract as well. The rigid periderm, however, remains fixed and sets the maximum diameter for stolonal expansion. In cross section, the stolon (and lumen) is not cylindrical but more closely resembles a half ellipse with the "flat" surface adjacent to the substratum. During tissue expansion and contraction in response to flow, the lumen cross section may change shape, thereby complicating the physical biology of fluid flow (see Blackstone, 1996; Van Winkle and Blackstone, 1997).

Colony development from a primary polyp can be mimicked by surgically explanting 1–2 polyps from a colony onto a new surface. In both cases, *P. carnea* develops by lineal extension of the stolons, initiation of new stolonal tips, and iteration of feeding polyps on the stolons, and forms a loose network of polyps and stolons typical of many runner-like forms. Once the available substratum is covered, *P. carnea* colonies increase polyp and stolonal tip formation, producing a more closely knit network of stolons and ultimately initiating the sexual (medusoid) phase of the life cycle. When genetically identical clonal explants of the same colony are used, the development of treated colonies can be manipulated relative to control colonies, producing more sheet-like *P. carnea* and thus providing an experimental system to generally examine heterochronic variation in runners and sheets (Fig. 1).

Production of colonies and culture conditions

Colonies on hermit crab shells were collected from the Yale Peabody Museum Field Station in Connecticut

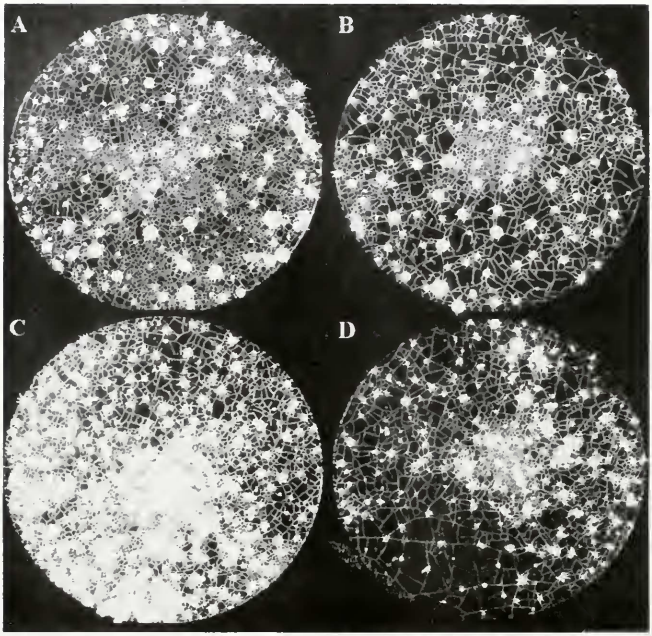

Figure 1. Background-subtracted images of several treated and control colonies of *Podocoryne carnea*: (A) treated with 30 μM dinitrophenol; (B) dinitrophenol control; (C) fed 6 times per week; (D) feeding control. The genetically identical colonies encrust 15-mm diameter glass cover slips and were imaged at the time of the initiation of medusa production (data from these images are included in Figs. 3 and 6). In each image, polyps are bright and circular, stolons are darker and web-like, and the substratum appears black. Both increased feeding and uncoupling increase the numbers of polyps and encrusting stolons at this developmental landmark; such colonies can be considered peramorphic; *i.e.*, they exhibit "shapes beyond" the normal developmental pathways.

Clonal replicates of one *P. carnea* colony were made by explanting 1–2 polyps and their connecting stolons onto round glass cover slips. For measures of flow rate and morphology, 15-mm diameter cover slips are ideal, but for measures of oxygen uptake and redox state, 12-mm diameter cover slips are necessary. After the explants attached and began to grow, all the original explant tissue was removed from the cover slip. In all experiments except the ones measuring redox state, colonies were effectively confined to one side of the cover slips by cutting back encrusting stolons from the reverse side on a daily basis. Cover slips were suspended in floating racks and grown in 120-liter aquaria containing Reef Crystals artificial seawater (salinity = 35‰) with temperature control to 20.5° ± 0.5°C, undergravel filtration, and 50% water changes weekly. Ammonia, nitrites, and nitrates were maintained below detectable levels (Aquarium Systems test kits). With the exception of the feeding manipulation experiments, colonies were fed to repletion with brine shrimp nauplii 3 days per week. Analysis has shown that, with similar culture conditions, "random" statistical effects (*e.g.*, time effects, tank effects, rack effects; see Sokal and Rohlf, 1981) are negligible (Blackstone and Buss, 1991).

Colony image analysis and morphometrics

Colonies were measured using image analysis technology (see Rohlf and Bookstein, 1990). Briefly, a high-resolution MTI CCD-72 camera attached to a macro lens was used to project an image of each colony onto a color monitor interfaced with a PC compatible microcomputer (pentium/90 MHz CPU, 32 Mb RAM) equipped with an Overlay Frame Grabber board (640 × 480 pixels with 12-bit depth per pixel). OPTIMAS software was used to acquire background-subtracted images of the col-

onies with illumination appropriate to produce three distinct luminance thresholds: the polyps (lightest), the stolons (intermediate), and the empty cover slip (darkest). Using these thresholds, the software identified and measured the areas of the empty (*i.e.*, unencrusted) cover slip and of the individual polyps (Blackstone and Buss, 1992). Classification macros were used to identify and exclude areas of cover slip outside the edge of the colony. The total colony area and perimeter were also measured.

Data files were analyzed using PC-SAS software. For each colony at each measurement time, the total area of polyps and the total area of empty, unencrusted cover slip enclosed within the colony were expressed as a fraction of the total area (note that the total area of stolons can be calculated as 1 minus this combined fraction). Polyp area is clearly a measure of polyp development; empty, unencrusted inner area is largely a measure of stolon development. Although polyps can shield empty, unencrusted area from observation and measurement, in practice this is a minor source of error because stolon development is generally most extensive at the base of the polyps. This is particularly true at the time of initiation of the sexual (medusoid) phase of the life cycle and subsequently. Thus, at the times in development when morphology was measured (see below), polyp area and unencrusted inner area behave as largely independent measures of two different aspects of development. Morphological differences between experimental treatments were assessed by two methods: first, using an analysis of variance with the outcome equal to the natural logarithm of the ratio of total polyp area divided by total unencrusted inner area; second, using a multivariate analysis of variance with two outcomes, the natural logarithm of the ratio of total polyp area divided by total colony area and the natural logarithm of the ratio of total unencrusted inner area divided by total colony area. (Natural logarithms were used to better meet the assumptions of parametric statistics.) Both analyses ask essentially the same questions in slightly different ways, and both give very similar results. To avoid redundancy, only the results of the MANOVA's are reported here. Additional analyses using other measures of morphology (*e.g.*, the ratio of total polyp area to mean inner area; see Blackstone and Buss, 1992, 1993) provide very similar between-treatment effects.

Video microscopic measures of peripheral gastrovascular flow

Gastrovascular flow reaches a maximum 2–8 h after feeding (Schierwater *et al.*, 1992); all of these studies were carried out 3–5 h after feeding. The colony was placed in a flow-through chamber with a #1 cover slip as its base (Warner Instruments). The temperature of the inflowing seawater was adjusted with a thermoelectric device to maintain a constant chamber temperature (20.5° ± 0.3°C; chamber temperature was monitored with a YSI cuvette thermometer with a flexible probe). Colonies were viewed on an inverted light microscope (Zeiss Axiovert 135), with a 40× Plan-Neo objective in differential interference contrast. Three primary stolon tips from each colony were videotaped with the MTI CCD camera for 10 minutes each.

Gastrovascular flow must be reversed in each distal, "dead-end" tip. Stolon tips fill as fluid enters; the velocity of the fluid then decreases to zero (Fig. 2). Tips then empty, and the fluid velocity again decreases to zero. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time. These width or diameter measures are taken at the base of the lumen. With the image analysis system connected to the VCR, the diameter of the stolon lumen was measured at a point ~250 μm behind the tip itself. In this region of the stolon, gastrovascular fluid velocity goes to zero as the lumen diameter approaches its maximum and minimum (thus velocity itself need not be measured). Lumen diameter was measured when the stolon was full and when it was empty, for 3 consecutive, but nonoverlapping, cycles. For each cycle, the net lumen amplitude, that is, the difference between the maximum and minimum lumen diameters, was calculated. Periderm-to-periderm total stolon width (which is invariant throughout the contraction cycle) and the period (in seconds) of each cycle were also measured (Fig. 2).

Statistical analysis focused on the three measured outcomes: lumen amplitude, cycle period, and stolon width. The interpretation of these measures in terms of the volumetric rate of gastrovascular flow has been discussed in detail elsewhere (Blackstone, 1996). Briefly, these measures can be combined into a biologically meaningful measure of gastrovascular flow rate: lumen amplitude divided by cycle period and stolon width (micrometers of lumen diameter per total micrometers of stolon width per second). Biologically, this rate measure illuminates the rate of supply of food to the tissues of the stolon tip. Both this rate measure and the individual flow parameters generally meet the assumptions of parametric statistics (see Sokal and Rohlf, 1981). To compare treatments, a nested analysis of variance was used with cycles nested within stolons, stolons nested within colonies, and colonies nested within treatments. In some cases, near-significant effects that may be biologically relevant are discussed (for justification, see Rothman, 1986).

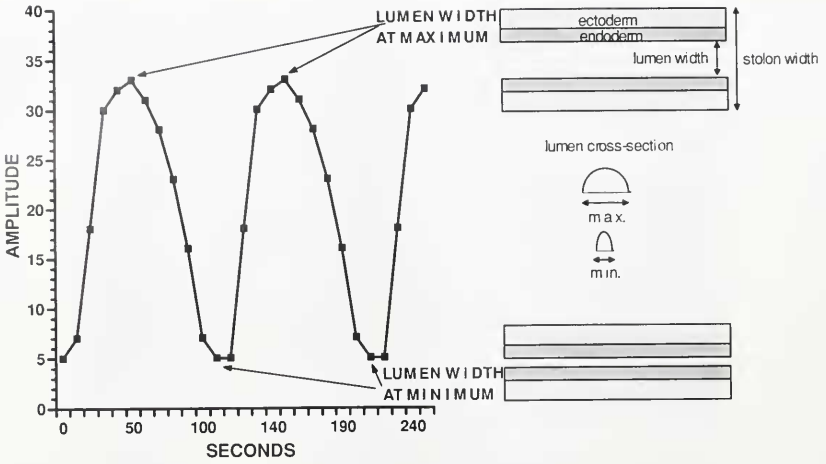

Figure 2. The contraction and expansion of the stolon lumen as it fills and empties largely in response to gastrovascular flow. Amplitude for each cycle corresponds to the diameter of the lumen. Schemata show transverse sections and cross sections of the stolon tip with the lumen at maximal and minimal width. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time (modified from Blackstone, 1996).

Feeding manipulation experiments

Eighteen newly explanted clonal replicates on 15-mm cover slips were randomly divided into three groups. Each group received a different number of feedings per week (2×, 3× [control], and 6×). The morphology of each colony was measured at the time the substratum became covered, when medusa production was initiated, and when the first medusae were released. Immediately preceding the first measure, gastrovascular flow to three stolon tips per colony was measured. The initiation of medusa production was defined as the day after the first medusa buds became visible on gonozooids of the colony. The time of the first release of medusae was defined as the day after maturing medusae (*e.g.*, tentacles extended and swimming bell fully extended and contractile) were first observed on gonozooids of the colony.

Uncoupling experiments

Eighteen newly explanted clonal replicates on 15-mm cover slips were randomly divided into three groups. Each group was treated with a different concentration of 2, 4-dinitrophenol in seawater (0 μM [control], 15 μM, and 30 μM) for 4 h per day. Such treatment with dinitrophenol results in "loose-coupling" of oxidative phosphorylation, oxidation of NADH, and diminished ATP formation (Blackstone and Buss, 1992, 1993). Dinitrophenol treatment was carried out in glass petri dishes containing 50 ml of the appropriate solution. Each colony was kept in a single petri dish. Dishes were arranged on three trays (one for each treatment) in an incubator (20.5°C). To randomize position and shelf effects, each colony's dish was shifted by one position each day, and trays were moved to a different shelf each day. Petri dishes were changed daily. When colonies were not being treated with dinitrophenol, they were kept in the aquaria in the normal fashion. Colony morphology and gastrovascular flow rate were measured at the same developmental landmarks as in the feeding manipulation experiments.

Dose-response relationships

In evaluating dose-response relationships between physiological parameters (gastrovascular flow rate in this case) and a morphological outcome, several considerations arise. A considerable difference is expected in the range of values between a physiological dose and a morphological response. A physiological measure is relatively instantaneous, whereas a morphological measure is cumulative. Hence, even a small physiological difference can, over time, produce a large difference in mor-

phology. The same logic suggests that this sort of dose-response relationship can best be evaluated by measures that are offset in time. In other words, the morphological effect of a physiological dose measured at one time should be assessed by morphological measures taken at later times. Given these considerations, the mean of nine measures (from three cycles of three stolons) of gastrovascular flow taken from each colony at the time the surface became covered was used to predict morphological outcomes taken subsequently (*i.e.*, at the initiation of medusa production and at the first release of medusae).

Dose-response relationships were assessed graphically and by using parametric and nonparametric measures of correlation. Gastrovascular flow rate (*i.e.*, lumen amplitude divided by cycle period and stolon width) was used as the predictor, and the ratio of total polyp area to total unencrusted inner area was used as the morphological outcome (the results were found to be insensitive to the particular morphological metric used). Both measures were natural log transformed to better meet the assumptions of the parametric correlations. However, particularly in the case of the overfeeding experiments, the assumption of a bivariate normal distribution is not well satisfied. Although all measures of correlation were similar, in this case the nonparametric correlations are most appropriate, and only Spearman's R_s are reported here (Sokal and Rohlf, 1981). Correlations between individual flow parameters (lumen amplitude, cycle period, and stolon width) and the morphological outcome were also tested.

Finally, it should be noted that the dose-response relationships for the feeding manipulation and the uncoupling experiments may not be directly comparable. In particular, previous workers have shown that confining colonies in petri dishes can lead to more sheet-like growth (Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991). In such confined cultures, soluble morphogenetic factors produced by the colonies can accumulate and enhance polyp and stolon production. The dinitrophenol experiments include this effect in all three treatments and are thus not directly comparable to the entirely aquarium-grown colonies of the feeding manipulation experiments.

Measures of oxygen uptake

Five newly explanted clonal replicates were allowed to grow on 12-mm cover slips for 2 weeks. At that point, the effect of 30 μM dinitrophenol on the rate of oxygen consumption of each colony was measured. First, a cover slip, with its colony attached, was affixed with a drop of silicone grease to a cover slip cemented to a small magnet. This assembly was contained in a 13-mm diameter sealed glass chamber (Strathkelvin RC300) with 0.7 ml of seawater (filtered to 0.2 μm). Chamber temperature was held constant (20.5° ± 0.02°C) by means of an external circulation water bath (Neslab RTE-100D), and the rate of decline in oxygen concentration over a 30-min period was measured (using a Strathkelvin 1302 electrode and 781 oxygen meter) with stirring (by slowly spinning the magnet, cover slips, and colony). Subsequently, the chamber was opened, 0.021 ml of seawater removed, 0.021 ml of 1 mM dinitrophenol solution in seawater added, the solution mixed and aerated thoroughly with a small pipette, and the chamber resealed (this procedure took ~7 min). The rate of decline in oxygen concentration was then measured for another 30 min. Experiments were performed 3–5 h after the subject colony was fed, as part of the normal feeding schedule. For the five colonies, the entire experiment thus took nearly 2 weeks.

After each colony was tested, a control experiment was carried out with the same five colonies and an identical protocol, except that instead of adding uncoupler, plain seawater was added prior to the second measure of the decline in oxygen concentration. Following these control experiments, feeding experiments were done, again with the same five colonies on the normal feeding schedule. In this case, a similar protocol was used, except that after the first 30-min measure of the decline in oxygen concentration, the colony was removed from the chamber, fed a small amount of brine shrimp, carefully cleaned of any shrimp or shrimp portions remaining outside the gastrovascular cavity, and returned to the chamber, which was then resealed (this procedure took ~20 min). The decline in oxygen concentration was then measured again. In each of these experiments, data were analyzed using a paired-comparison t test.

Measures of mitochondrial redox state

Spectrofluorometric assays of NAD^+/NADH provide a useful measure of mitochondrial redox state (*e.g.*, Chance and Baltscheffsky, 1958; Chance and Thorell, 1959; Chance *et al.*, 1963; Chance, 1991). These measures are carried out *in vivo*, with no apparent damage to the colonies, using a Perkin-Elmer spectrofluorometer (excitation at 366 nm and emission read near 460 nm) and acrylic cuvettes (which do not absorb or emit when excited above 300 nm). Similar experiments with a variety of clones of different hydroid species (including the *P. carnea* clone used here) have already demonstrated that uncoupling with 30 μM dinitrophenol triggers a shift of the redox state in the direction of oxidation (Blackstone and Buss, 1992, 1993; Blackstone, in press). Here, the effects of feeding were investigated. Eighteen newly explanted clonal replicates were allowed to completely cover both sides of 12-mm cover slips

(~2 months). To produce an adequate signal, three colonies on cover slips were read simultaneously. Thus the effective sample size is $n = 6$. The emission of each group was measured; the colonies were then removed from the cuvette, fed a small amount of brine shrimp, carefully cleaned of any shrimp or shrimp portions remaining outside the gastrovascular cavity, and returned to the cuvette for a second measurement of emission. A control experiment was performed with the same colonies and an identical protocol, except that colonies were not fed between emission readings. Data were analyzed using paired-comparison t tests.

Results

Feeding manipulation experiments

Representative colony images can be seen in Figure 1 and on the World Wide Web (http://www.bios.niu.edu/eande/blackstone/blackstone.html). Feeding manipulation substantially affects colony morphology such that higher rates of feeding produce colonies with a greater area of polyps and a lesser unencrusted area within the colony (Fig. 3). At the time they completely covered the surface, colonies in each treatment are roughly similar, but there is a small between-treatment effect (MANOVA, $F = 2.93$, df = 4, 28, $P < 0.05$). Subsequently in colony development, the differences between treatments become profound (Fig. 3; MANOVA at the initiation of medusa development, $F = 13.2$, df = 4, 28, $P \ll 0.001$; MANOVA at the first release of medusae, $F = 24.7$, df = 4, 28, $P \ll 0.001$). At the time of covering the surface, there is also a significant between-treatment difference in flow rate (Fig. 4; using the colonies-within-treatment effect as the error term, $F = 8.9$, df = 2, 15, $P < 0.01$) such that a higher rate of feeding diminishes the flow. Since flow rate is a composite of three measured flow parameters (lumen amplitude, cycle period, and stolon width), it is illuminating to examine the between-treatment differences in these variables individually. Lumen amplitude and cycle period show no significant differences (Fig. 5; $F = 0.20$, df = 2, 15, $P > 0.8$, and $F = 1.43$, df = 2, 15, $P > 0.25$, respectively for each variable using the colonies-within-treatment effect as the error term). Stolon width, however, shows a dramatic between-treatment difference (Fig. 5; $F = 23.8$, df = 2, 15, $P \ll 0.001$, again using the colonies-within-treatment effect as the error term). A higher rate of feeding produces thicker stolons, and the between-treatment differences in flow rate derive not from differences in the amount of flow (amplitude and period), but from differences in the thickness of the stolons.

Uncoupling experiments

Representative colony images can be seen in Fig. 1 and on the World Wide Web (at the URL given in the previous section). Treatment with dinitrophenol affects colony morphology such that higher concentrations produce colonies with a lesser unencrusted area within the colony and a greater area of polyps (Fig. 6). At the time of covering the surface, colonies in each treatment are similar (MANOVA, $F = 2.0$, df = 4, 28, $P > 0.10$). Later in colony development, the differences between treatments become pronounced (Fig. 6; MANOVA at the initiation of medusa development, $F = 9.94$, df = 4, 28, $P \ll 0.001$; MANOVA at the first release of medusae, $F = 8.54$, df = 4, 28, $P \ll 0.001$). At the time of covering the surface, there is also a significant between-treatment

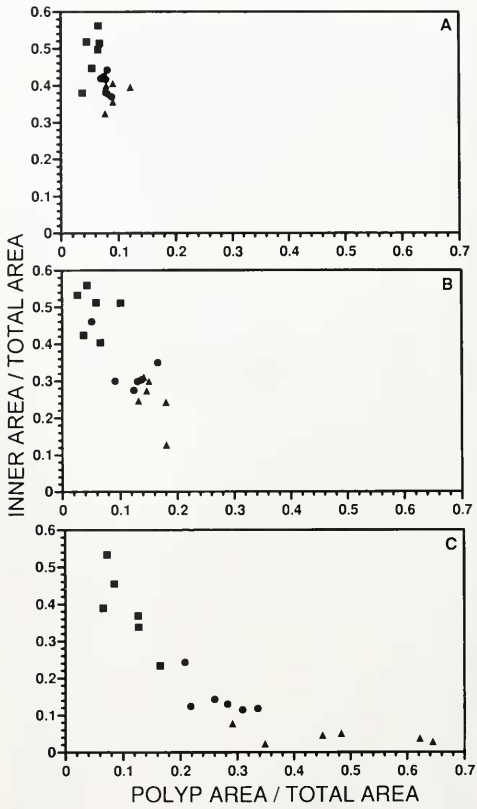

Figure 3. Bivariate scatterplots for colonies of three feeding treatments at three stages of development. Treatment: (A) 2× per week; (B) 3× per week; (C) 6× per week. Stage: squares = covering the surface; circles = initiation of medusa production; triangles = first release of medusae. The y-axis is the total area of unencrusted surface enclosed within the colony; the x-axis is the total area of polyps for each colony. Both measures are adjusted for colony size.

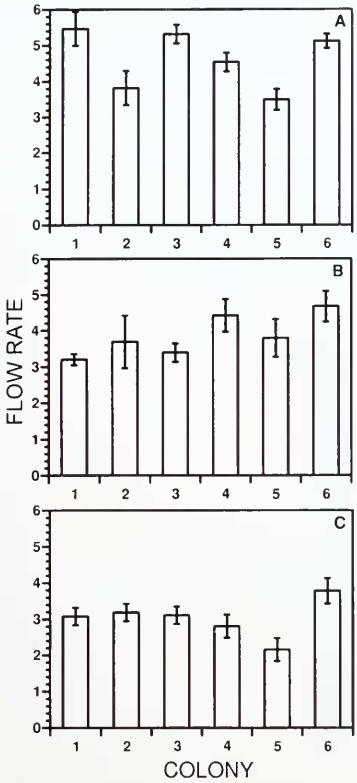

Figure 4. Means and standard errors of flow rates times 10^3 (μm of lumen expansion and contraction per total μm of stolon width per second) for the six colonies of each of three feeding treatments: (A) 2× per week, (B) 3× per week, (C) 6× per week. Colonies of the three treatments are numbered arbitrarily.

difference in flow rate (Fig. 7; using the colonies-within-treatment effect as the error term, $F = 13.4$, df = 2, 15, $P < 0.001$). Treatment with dinitrophenol diminishes the flow rate. As in the feeding experiments, the between-treatment differences in these variables were examined individually. Lumen amplitude and cycle period show significant or near-significant differences (Fig. 8; $F = 7.1$, df = 2, 15, $P < 0.01$, and $F = 3.54$, df = 2, 15, $P < 0.06$, respectively for each variable using the colonies-within-treatment effect as the error term). Stolon width, however, shows no difference (Fig. 8; $F = 1.34$, df = 2, 15, $P > 0.25$, again using the colonies-within-treatment effect as the error term). Treatment with dinitrophenol primarily diminishes the lumen amplitude and slightly increases the cycle period; the between-treatment differences in flow rate derive from differences in the amount of flow (amplitude and period), not from differences in the thickness of the stolons.

Dose-response relationships

Both experiments produced significant dose-response relationships for flow rate and morphological measures (Fig. 9). Lower flow rates produced greater polyp areas and lesser amounts of unencrusted inner areas. For the feeding manipulation experiments at the time of the initiation of medusa production (Fig. 9a), $R_S = -0.59$ ($P < 0.01$). For the feeding manipulation experiments at the time of the first release of medusae (Fig. 9b), $R_S = -0.69$ ($P < 0.001$). For the uncoupling experiments at the corresponding developmental stages (Fig. 9c and 9d), $R_S = -0.68$ ($P < 0.01$) and $R_S = -0.69$ ($P < 0.001$). There may be an effect of the small culture dishes used in the uncoupling experiments (see Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991) such that the control colonies in these experiments have greater amounts of polyp area and lesser amounts of unencrusted inner area than their counterparts in the feeding manipulation experiments. Nevertheless, for the colonies of each treatment in which flow was most perturbed (triangles on each plot), a similar flow rate produces a similar morphology.

The dose-response relationships of individual flow parameters and morphological measures correspond to the overall between-treatment effects (Figs. 5 and 8). For the feeding manipulation experiment, stolon width is highly correlated with the morphological outcome ($R_S = 0.78$, $P \ll 0.001$ and $R_S = 0.77$, $P \ll 0.001$, for the two developmental stages respectively). There are no significant correlations for lumen amplitude ($R_S = 0.16$, $P > 0.5$ and $R_S = 0.05$, $P > 0.8$, for the two developmental stages respectively) and cycle period ($R_S = 0.19$, $P > 0.45$ and $R_S = 0.34$, $P > 0.15$, for the two developmental stages respectively). For the uncoupling experiment, lumen amplitude is highly correlated with the morphological outcome ($R_S = -0.69$, $P < 0.001$ and $R_S = -0.78$, $P \ll 0.001$, for the two developmental stages respectively), and there are no significant correlations for stolon width ($R_S = 0.4$, $P > 0.1$ and $R_S = 0.07$, $P > 0.8$, for the two developmental stages respectively) and cycle period ($R_S = 0.27$, $P > 0.25$ and $R_S = 0.37$, $P > 0.1$, for the two developmental stages respectively).

Measures of oxygen uptake

Both uncoupling and feeding triggered an increase in the rate of decline of the oxygen concentration for each colony in the sealed chamber (Fig. 10). In the uncoupling experiments, for the five colonies the mean before/after

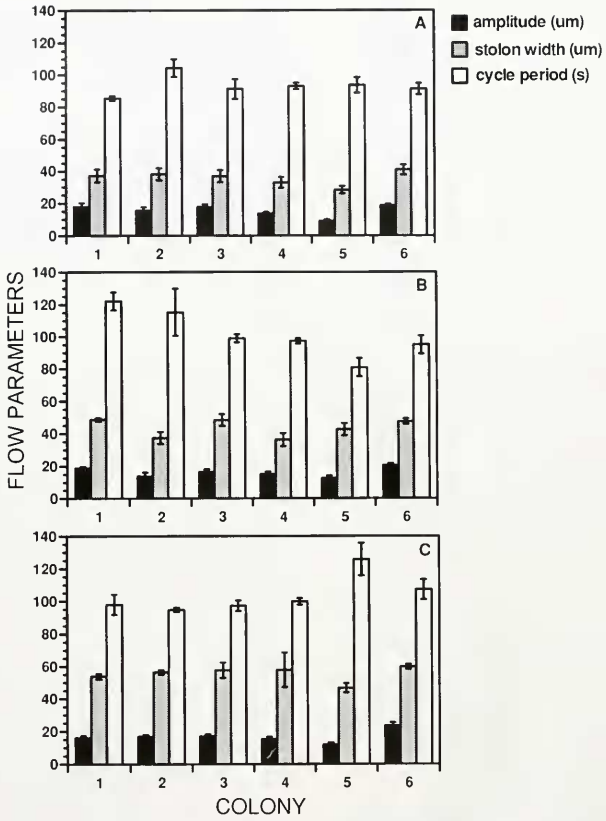

Figure 5. Means and standard errors for the three flow parameters that provide the rate measures in Figure 4 for the six colonies of each of three feeding treatments. Treatment: (A) 2× per week; (B) 3× per week; (C) 6× per week. Flow parameters: filled bars = lumen amplitude in μm; hatched bars = stolon width in μm; open bars = cycle period in seconds. Colonies of the three treatments are numbered arbitrarily.

difference in the slope of oxygen concentration versus time (± standard error) = −0.008 ± 0.0017, and this difference is significantly different from 0 (paired comparison t test, $t = -4.5$, $P < 0.01$). In the feeding experiments, for the five colonies the mean difference = −0.041 ± 0.0048, and this difference is again significantly different from 0 (paired comparison t test, $t = -8.4$, $P < 0.001$). Note that in the latter experiments, the five colonies were about 1 month older and significantly larger than in the former. Control experiments, carried out in an identical manner with no manipulation, showed a before/after difference that was positive (mean difference = 0.004 ± 0.001, paired comparison t test, $t = 4.2$, $P < 0.01$). It is likely that confinement in a small chamber results in a build-up of waste products and eventually inhibits colony metabolism (see discussion in Hoegh-Guldberg and Manahan, 1995); thus the increase in oxygen consumption detected in the uncoupling and feeding experiments is probably a conservative measure of the actual effect.

Measures of mitochondrial redox state

After feeding, the peak height at 460 nm declined by an average of 42% (using units of relative intensity, the

mean before/after decline ± standard error = 4.7 ± 0.6, paired comparison t test, $t = 7.8$, $P < 0.001$). Under the same protocol but with no feedings between the emission readings, this peak height increased by an average of less than 1% (the mean before/after increase ± standard error = 0.02 ± 0.13, paired comparison t test, $t = 0.13$, $P > 0.9$). Since this peak corresponds to mitochondrial NADH, the data suggest that feeding caused a significant oxidation of this NADH as measured by spectrofluorometry. These results suggest an overall shift in the mitochondrial redox state in the direction of oxidation (Chance, 1991) and parallel the effect observed with treatments of uncouplers (Blackstone and Buss, 1992, 1993; Blackstone, in press).

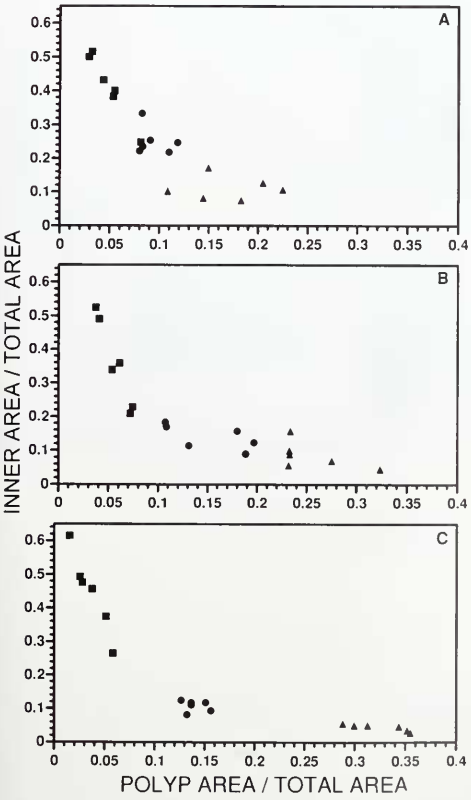

Figure 6. Bivariate scatterplots for colonies of three dinitrophenol treatments at three stages of development. Treatment: (A) 0 μM, (B) 15 μM, (C) 30 μM. Stage: squares = covering the surface; circles = initiation of medusa production; triangles = first release of medusae. The y-axis is the total area of unencrusted surface enclosed within the colony; the x-axis is the total area of polyps for each colony. Both measures are adjusted for colony size.

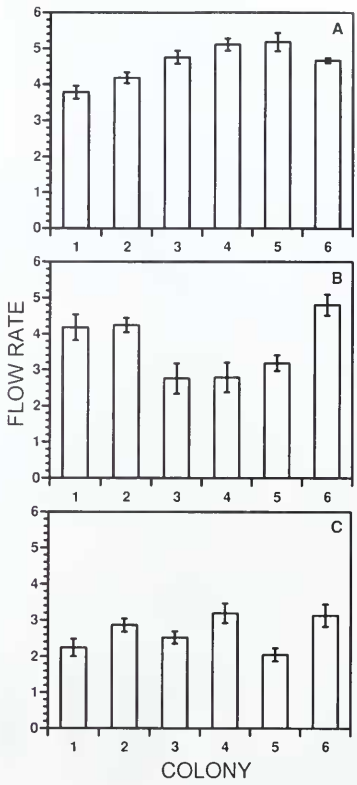

Figure 7. Means and standard errors of flow rates times 10^3 (μm of lumen expansion and contraction per total μm of stolon width per second) for the six colonies of each of three dinitrophenol treatments: (A) 0 μM, (B) 15 μM, (C) 30 μM. Colonies of the three treatments are numbered arbitrarily.

Discussion

Both the feeding manipulation and the uncoupling experiments suggest that there is a dose-response relationship between the measures of gastrovascular flow to peripheral stolons and the timing of colony development in *P. carnea*. Such a biological gradient provides strong evidence for a causal relationship between these two variables (see Hill, 1965; Weed, 1988). Some differences in experimental protocols were necessary for the two experiments; in particular, treatment with dinitrophenol required confining the colonies in petri dishes. In such con-

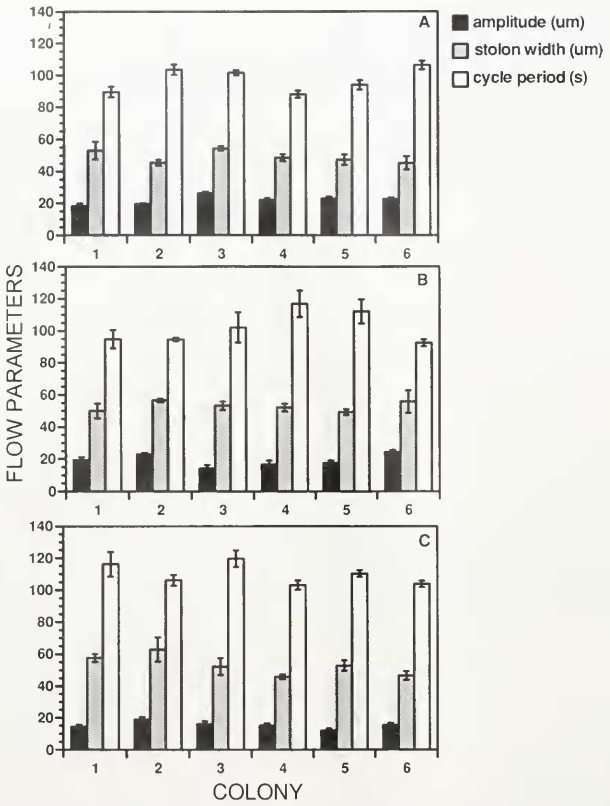

Figure 8. Means and standard errors for the three flow parameters that provide the rate measures in Figure 7 for the six colonies of each of three dinitrophenol treatments. Treatment: (A) 0 μM, (B) 15 μM, (C) 30 μM. Flow parameters: filled bars = lumen amplitude in μm; hatched bars = stolon width in μm, open bars = cycle period in seconds. Colonies of the three treatments are numbered arbitrarily.

fined cultures, soluble morphogenetic factors produced by the colonies can accumulate and enhance polyp and stolon production (Müller *et al.*, 1987; Plickert *et al.*, 1987; Lange and Müller, 1991). This may explain the difference between the dose-response curves in the two experiments. However, the effects of the confined cultures are most pronounced in the control colonies, whereas in the colonies in which flow was most diminished, a similar rate of flow produced a similar morphological response in both experiments. This result suggests that the rate of gastrovascular flow and the morphogenetic factors that act in confined cultures (*e.g.*, stolon-inducing factor, a 20-kDa glycoconjugate; see Lange and Müller, 1991) may interact, although the nature of this interaction remains unclear, and alternative explanations are possible as well (see below).

Although the feeding manipulation and the uncoupling experiments both suggest that morphology is mediated by the rate of gastrovascular flow, it is clear that these treatments differ with respect to the actual parameters that underlie flow rate. Feeding manipulation strongly affects the thickness of the stolons. Underfed colonies have very narrow stolons with insubstantial tissue layers, but the actual amount of flow (as measured by lumen amplitude and cycle period) is not affected. In contrast, overfed colonies have wide stolons with thick tissue layers, and again the amount of flow is unaffected. In this way, the stolons of underfed colonies receive more

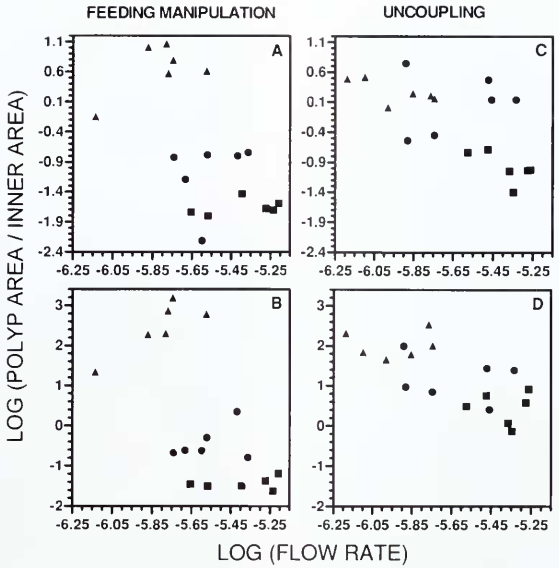

Figure 9. Dose-response relationships for feeding manipulation experiments (A, B) and uncoupling experiments (C, D). In the former, the feedings per week were manipulated (triangles = 6×, circles = 3×, and squares = 2×); in the latter, each group was treated with a different concentration of 2, 4-dinitrophenol in seawater (triangles = 30 μM, circles = 15 μM, and squares = 0 μM) for 4 h per day. (A) Feeding experiment at the initiation of medusa production; (B) feeding experiment at the first release of medusae; (C) uncoupling experiment at the initiation of medusa production; (D) uncoupling experiment at the first release of medusae.

flow relative to their size than do the stolons of overfed colonies. Uncoupling, however, strongly affects the actual amount of flow, particularly as measured by the lumen amplitude. Stolons of treated colonies have diminished lumen amplitudes relative to controls, but the thickness of the stolon is unchanged. Thus it is in this alternative way that uncoupling diminishes the flow rate relative to the size of the stolon. A plausible conclusion is that the increased feeding and the uncoupling experiments affect flow by very different mechanisms—the former by a general thickening of the stolon, perhaps increasing the stolon's resistance to flow or its absorption of gastrovascular fluid, and the latter by diminishing the capability of the polyps to pump fluid.

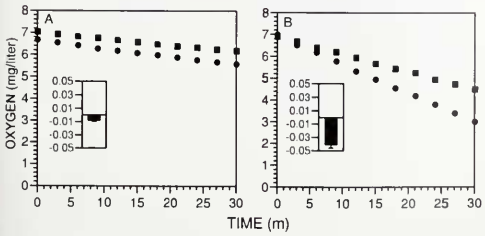

Figure 10. Rate of decline in oxygen concentration for (A) a colony before (squares) and after (circles) treatment with an uncoupler (dinitrophenol at 30 μM,) and (B) a colony before (squares) and after (circles) feeding. For five colonies, inset plots show the mean ± standard error of the before/after difference in the rate of decline in oxygen concentration, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. For both experiments, this difference in rate was significantly negative; *i.e.*, with both treatments the oxygen uptake of the colony increased after the treatment.

On the other hand, both uncoupling and feeding produce some similarities at the level of cellular metabolism. Both treatments trigger aspects of metabolic activation as measured by oxygen uptake and mitochondrial redox state. The possible effects of metabolic state on colony development remain largely unexplored. Earlier this century, prominent hydroid biologists considered metabolic gradients to be a principal determinant of development (*e.g.*, Child and Hyman, 1919; Child, 1941). Although these suggestions have generally been replaced by the theory of variable gene activity (*e.g.*, Davidson, 1968), work on hydroids has continued to implicate me-

tabolism in development (*e.g.*, Newman, 1973; May and Müller, 1975). Child's theories of metabolic gradients (see Mitman and Fausto-Sterling, 1992) may yet be reconciled with the theory of variable gene activity (*e.g.*, Nijhout, 1990; Blackstone, 1997).

In these hydroids, it is further possible that there may be an interaction between flow rate and metabolic state. Gastrovascular flow distributes food throughout the colony, and such substrate has been shown to affect mitochondrial metabolic state. The dynamics of the stolon endoderm (*e.g.*, Schierwater *et al.*, 1992) may mediate the relationship between flow rate and food uptake, and this relationship may not be straightforward or simple. The interaction between flow rate and metabolic state is thus another possible determinant of the timing of colony development. This interaction provides another possible explanation for the somewhat different dose-response curves obtained for the uncoupling experiments as compared to the feeding manipulation experiments.

In summary, the dose-response relationships between flow rate and colony development strongly suggest that the timing of polyp and stolon tip formation in these hydroids is mediated by features of the gastrovascular system. Nevertheless, the data on metabolic activation point out the complexities inherent in analyzing experimental perturbations of this system. Since gastrovascular flow supplies substrate to the stolon tissues, any perturbation of flow alters not only the physical characteristics of the flow, but the supply of substrate as well. The latter may affect metabolic state and may act together with or separately from flow in determining the timing of polyp and stolon tip formation. In addition, the dynamics of the stolon endoderm add another layer of complexity to the effects of flow rate and substrate supply. Finally, these physiological and metabolic aspects of the stolon must ultimately interact with pattern-forming genes (*e.g.*, Kuhn *et al.*, 1996; Cartwright, 1997) in as-yet-undetermined ways to trigger the morphogenetic events associated with polyp and stolon tip formation.

Additional experiments are clearly necessary. Crucially, these experiments must effectively distinguish between the flow rate and metabolic state *in vivo*. A variety of optical methods (*e.g.*, Chance, 1991) and computer-automated video microscopy (Van Winkle and Blackstone, 1997) can be used to visualize redox state and flow rate simultaneously in a living stolon; then after various perturbations are applied, the rates of polyp and stolon tip development can be measured in that stolon. Such an experimental system is being developed with the expectation of better resolving the roles of flow rate and metabolic factors in the development and heterochrony of these hydroids and of other colonial animals as well.

Acknowledgments

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Literature Cited

Alberch, P., and E. Gale. 1985. A developmental analysis of an evolutionary trend: digital reduction in amphibians. *Evolution* **39:** 8–23.

Alberch, P., S. J. Gould, G. F. Oster, and D. B. Wake. 1979. Size and shape in ontogeny and phylogeny. *Paleobiology* **5:** 296–313.

Blackstone, N. W. 1996. Gastrovascular flow and colony development in two colonial hydroids. *Biol. Bull.* **190:** 56–68.

Blackstone, N. W. 1997. Individuality in early eukaryotes and the consequences for metazoan development. *Prog. Mol. Subcell. Biol.* **19** (in press).

Blackstone, N. W. Physiological and metabolic aspects of experimental heterochrony in colonial hydroids. *J. Evol. Biol.* **10** (in press).

Blackstone, N. W., and L. W. Buss. 1991. Shape variation in hydractiniid hydroids. *Biol. Bull* **180:** 394–405.

Blackstone, N. W., and L. W. Buss. 1992. Treatment with 2, 4-dinitrophenol mimics ontogenetic and phylogenetic changes in a hydractiniid hydroid. *Proc. Natl. Acad. Sci., USA* **89:** 4057–4061.

Blackstone, N. W., and L. W. Buss. 1993. Experimental heterochrony in hydractiniid hydroids: why mechanisms matter. *J. Evol. Biol.* **6:** 307–327.

Bonner, J. T., ed. 1982. *Evolution and Development.* Springer-Verlag, Berlin.

Braverman, M. 1974. The cellular basis for colony form in *Podocoryne carnea*. *Am. Zool.* **14:** 673–698.

Buss, L. W., and N. W. Blackstone. 1991. An experimental exploration of Waddington's epigenetic landscape. *Philos. Trans. R. Soc. Lond B* **332:** 49–58.

Cartwright, P. 1997. Characterization of a HOM/Hox type homeobox gene, *Cnox2*, in the colonial hydrozoan, *Hydractinia symbiolongicarpus*. Ph. D. Dissertation, Yale Univ., New Haven, CT.

Chance, B. 1991. Optical method. *Annu. Rev. Biophys. Biophys. Chem.* **20:** 1–28.

Chance, B., and H. Baltschelffsky. 1958. Respiratory enzymes in oxidative phorphorylation. *J. Biol. Chem.* **233:** 736–739.

Chance, B., and B. Thorell. 1959. Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry. *J. Biol. Chem.* **234:** 3044–3050.

Chance, B., G. R. Williams, and G. Hollunger. 1963. Inhibition of electron and energy transfer in mitochondria. *J. Biol. Chem.* **278:** 439–444.

Child, C. M. 1941. *Patterns and Problems in Development.* Univ. Chicago Press, Chicago.

Child, C. M., and L. H. Hyman. 1919. The axial gradients in Hydrozoa. *Biol. Bull.* **36:** 183–224.

Davidson, E. H. 1968. *Gene Activity in Early Development.* Academic Press, New York.

DeSalle, R., and E. Carew. 1992. Phyletic phenocopy and the role of developmental genes in morphological evolution in the Drosophilidae. *J. Evol. Biol.* **5:** 363–374.

Dudgeon, S. R., and L. W. Buss. 1996. Growing with the flow: on the maintenance and malleability of colony form in the hydroid *Hydractinia*. *Am. Nat* **147:** 667–691.

Gould, S. J. 1977. *Ontogeny and Phylogeny.* Harvard Press, Cambridge, MA.

Hall, B. K. 1992. *Evolutionary Developmental Biology.* Chapman and Hall, London.

Harper, J. L. 1985. Modules, branches and the capture of resources. Pp. 1–33 in *Population Biology and Evolution of Clonal Organisms,* J. B. C. Jackson, L. W. Buss, and R. E. Cook, eds. Yale Univ. Press, New Haven, CT.

Heytler, P. G. 1981. Uncouplers of oxidative phosphorylation. Pp. 199–210 in *Inhibitors of Mitochondrial Functions,* M. Erecinska and D. F. Wilson, eds. Pergamon Press, New York.

Hill, A. B. 1965. The environment and disease: association or causation? *Proc. R. Soc. Med.* **58:** 295–300.

Hoegh-Guldberg, O., and D. T. Manahan. 1995. Coulometric measurement of oxygen consumption during development of marine invertebrate embryos and larvae. *J. Exp. Biol.* **198:** 19–30.

Jackson, J. B. C. 1979. Morphological strategies of sessile animals. Pp. 499–556 in *Biology and Systematics of Colonial Organisms,* G. Larwood and B. Rosen, eds. Academic Press, London.

Ketterson, E. D., and V. J. Nolan. 1992. Hormones and life histories: an integrative approach. *Am. Nat.* **140:** S33–S62.

Kuhn, K., B. Streit, and B. Schierwater. 1996. Homeobox genes in the cnidarian *Eleutheria dichotoma:* evolutionary implications for the origin of *Antennapedia-class* (HOM/Hox) genes. *Mol. Phylogenet. Evol.* **6:** 30–38.

Lange, R. G., and W. A. Muller. 1991. SIF, a novel morphogenetic inducer in Hydrozoa. *Dev. Biol.* **147:** 121–131.

May, G., and W. A. Muller. 1975. Interpersonale Differenzierung des Enzymaktivitäts-Musters bei dem polymorphen Hydroiden *Hydractinia echinata. Wilhelm Roux' Archiv* **177:** 53–59.

McKinney, M. L., ed. 1988. *Heterochrony in Evolution: An Interdisciplinary Approach.* Plenum, New York.

Meyer, A. 1987. Phenotypic plasticity and heterochrony in *Cichlasoma managuense* (Pisces, Cichlidae) and their implications for speciation in cichlid fishes. *Evolution* **41:** 1357–1369.

Mitman, G., and A. Fausto-Sterling. 1992. Whatever happened to *Planaria?* C. M. Child and the physiology of inheritance. Pp. 172–197 in *The Right Tools for the Job,* A. E. Clarke and J. H. Fujimura, eds. Princeton Univ. Press, Princeton, NJ.

Muller, G. B. 1991. Experimental strategies in evolutionary embryology. *Am. Zool.* **31:** 605–615.

Muller, W. A., A. Hauch, and G. Plickert. 1987. Morphogenetic factors in hydroids: I. Stolon tip activation and inhibition. *J. Exp. Zool.* **243:** 111–124.

Newman, S. A. 1973. Reversible abolition of normal morphology in *Hydra. Nat. New Biol.* **244:** 126–128.

Nijhout, H. F. 1990. Metaphors and the role of genes in development. *BioEssays* **12:** 441–446.

Plickert, G., A. Heringer, and B. Hiller. 1987. Analysis of spacing in a periodic pattern. *Dev. Biol.* **120:** 399–411.

Raff, R. A., and G. A. Wray. 1989. Heterochrony: developmental mechanisms and evolutionary results. *J. Evol. Biol.* **2:** 409–434.

Rohlf, F. J., and F. L. Bookstein, eds. 1990. *Proceedings of the Michigan Morphometrics Workshop.* Univ. Michigan Mus. Zool., Ann Arbor, MI.

Rothman, K. J. 1986. Significance questing. *Ann. Intern. Med.* **105:** 445–447.

Schierwater, B., B. Pickos, and L. W. Buss. 1992. Hydroid stolonal contractions mediated by contractile vacuoles. *J. Exp. Biol.* **162:** 1–21.

Sinervo, B., and A. L. Basalo. 1996. Testing adaptation using phenotypic manipulations. Pp. 149–185 in *Adaptation,* G. Lauder and M. R. Rose, eds. Academic Press, New York.

Sinervo, B., P. Doughty, R. B. Huey, and K. Zamudio. 1992. Allometric engineering: a causal analysis of natural selection on offspring size. *Science* **258:** 1927–1930.

Sokal, R. R., and F. J. Rohlf. 1981. *Biometry.* Freeman, San Francisco.

Stebbins, G. L., and D. V. Basile. 1986. Phyletic phenocopies: a useful technique for probing the genetic and developmental basis of evolutionary change. *Evolution* **40:** 422–425.

Van Winkle, D. H., and N. W. Blackstone. 1997. Video microscopic measures of gastrovascular flow in colonial hydroids. *Invertebr. Biol.* **116:** 6–16.

Wake, D. B., P. M. Mabee, J. Hanken, and G. P. Wagner. 1991. Development and evolution—the emergence of a new field. Pp. 582–588 in *Unity of Evolutionary Biology,* E. Dudley, ed. Dioscorides Press, Portland, OR.

Weed, D. L. 1988. Causal criteria and Popperian refutation. Pp. 13–32 in *Causal Inference,* K. Rothman, ed. Journal of Epidemiology Resources, Inc., Chestnut Hill, MA.